

Lateral Diffusion of Wild-type and Mutant L<sup>d</sup> Antigens in L Cells

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**ABSTRACT** We have compared the lateral diffusion of intact transmembrane proteins, wild-type H-2L<sup>d</sup> antigens, with that of mutants truncated in the cytoplasmic domain. Diffusion coefficients and mobile fractions were similar for all molecules examined, from wild-type L<sup>d</sup> antigens with 31 residues on the cytoplasmic side of the plasma membrane to mutants with only four residues in the cytoplasmic domain. This result limits ways in which the lateral diffusion of a major histocompatibility antigen, a transmembrane protein, can be constrained by interactions with other molecules.

The lateral diffusion coefficients measured for membrane proteins range from ca.  $10^{-11}$  cm<sup>2</sup>s<sup>-1</sup> to  $5 \times 10^{-9}$  cm<sup>2</sup>s<sup>-1</sup> with values for many proteins  $\sim 2 \times 10^{-10}$  cm<sup>2</sup>s<sup>-1</sup>. This value is 10 to 20 times slower than the fastest diffusion observed, for rhodopsin in disc membranes, (14, 20, 29) and is much lower than expected on the basis of any theory for diffusion in membranes (5, 9, 22). Several lines of evidence suggest that the slow lateral diffusion of many membrane proteins is due to interactions between these proteins and the cytoskeleton: (a) Diffusion of band 3 is 100 times faster in spectrin-deficient mouse erythrocytes than in normal erythrocytes (13, 23). (b) Diffusion of several membrane proteins is increased 100-fold or more in membrane blebs produced on the surface of intact cells. Such blebs appear to lack actin (26, 28, 32). (c) Diffusion of membrane proteins reconstituted into synthetic lipid vesicles is orders of magnitude faster than diffusion of the same proteins in native membranes (17, 27).

Antigens specified by the mammalian major histocompatibility complex (MHC), class I MHC antigens, are expressed on most cells. Lateral diffusion of these antigens, H-2 of mouse and HLA of humans, has been studied in normal and transformed cells, (4, 6, 7, 18, 25) and in liposomes reconstituted with purified antigens (1). MHC antigens are, like other membrane integral proteins, somehow constrained in their lateral diffusion. Diffusion coefficients range from  $\ll 2$  to  $10 \times 10^{-10}$  cm<sup>2</sup>s<sup>-1</sup> in lymphocytes and in cultured fibroblasts, while they are  $50$ – $100 \times 10^{-10}$  cm<sup>2</sup>s<sup>-1</sup> for MHC antigens in liposomes (1).

A good deal is known about the structure of MHC class I antigens, and complete sequences are available for several different antigens (reviews in 8, 11). All class I antigens consist of a heavy chain of  $\sim 44,000$  d associated with a light chain, beta-2 microglobulin, of 12,000 d. The bulk of the complex lies outside of the cell membrane. A segment of 24 amino acids spans the membrane, and a segment of from 31 to 46 amino acids lies within the cell. This cytoplasmic domain

interacts with isolated proteins of the cytoskeleton (19) and other work suggests that the MHC antigens react with the cytoskeleton in intact membranes as well (12, 30). If this is the case, then mutant MHC molecules with modified or truncated cytoplasmic domains ought to diffuse more rapidly than wild-type antigens.

Truncated MHC genes have been produced by two laboratories. Zuniga et al. (33) transfected L cells with mutant H-2 L<sup>d</sup> genes which lacked as many as 24 of the 31 C terminal residues of wild-type L<sup>d</sup>. They showed that these mutant antigens were integrated in the membrane and that they effectively presented viral antigens to H-2L<sup>d</sup>-restricted cytotoxic T cells. Murre et al. (15) constructed a mutant L<sup>d</sup> in which all but six cytoplasmic residues were deleted and the deleted residues replaced by three amino acids specified by an MHC class II gene. These mutant genes were also expressed in transfected L cells and presented viral antigen to H-2 restricted killer cells. The truncated gene products also copped normally when reacted with anti-H-2 antibodies followed by antiglobulins. This implies that metabolically-driven lateral mobility of the truncated antigens is normal, but does not show if their diffusion differs from antigens with full-length cytoplasmic tails.

We have used fluorescence photobleaching to measure the lateral diffusion of wild-type and three mutant H-2 L<sup>d</sup> antigens expressed in L cells transfected with the appropriate genes. Three of these genes, the wild-type L<sup>d</sup> (27.5), a mutant (BAL907) in which 25 of the 31 residues of the cytoplasmic domain are replaced by 19 residues, not derived from the L<sup>d</sup> gene, and a mutant (BAL911) truncated to a seven residue cytoplasmic domain, have been described previously (33). A third mutant, C48, is further truncated to four residues of the wild-type cytoplasmic domain (lys-arg-ser-glu). The cytoplasmic sequences predicted for all 4 L<sup>d</sup> antigens are shown in Fig. 1.

Here we show that the diffusion coefficients of the four

# PREDICTED CYTOPLASMIC SEQUENCES OF MUTANT AND WILD TYPE L<sup>d</sup> MOLECULES

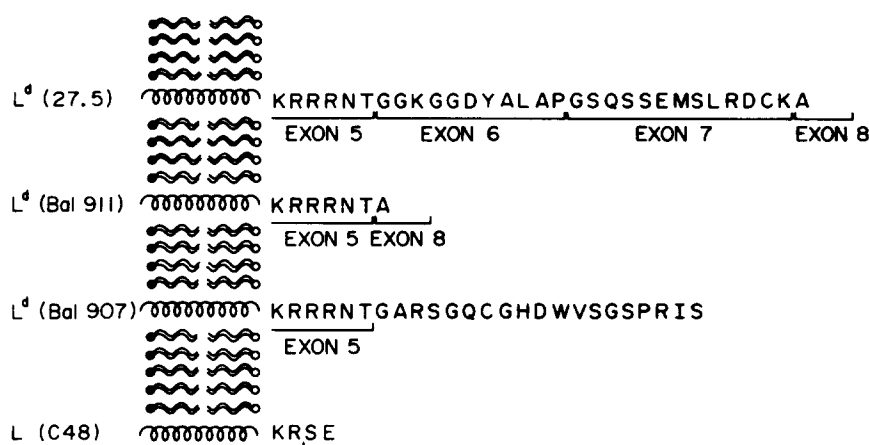


FIGURE 1 Cytoplasmic amino acid sequences predicted from the nucleotide sequences of the wild-type and three mutant L<sup>d</sup> genes. After (33) with permission. (Reproduced from *Cell*, 1983, 34:534-544, by copyright permission of MIT Press)

types of antigens differ less than twofold from one-another, and that the mobility of the mutant antigens appears to be restricted to the same extent and by the same factors as the mobility of wild-type H-2L and H-2K antigens.

## MATERIALS AND METHODS

Construction of mutants, transfection of mouse L cells, characterization and maintenance of transformants have been described previously (33). Mutant C48 was constructed in a similar manner; details of its construction will be published elsewhere (Zuniga, in preparation). Two clones of each transfectant were used in our experiments: 27.5.27 5-E-11 and 27.5.27 4-B-5, 911 B-6 and 911 D-6, 907 B-4 and 907 C-4, C48 1-F-5 and C48 1-C-4. No difference was found when diffusion coefficients were compared between clones expressing the same L<sup>d</sup> antigen and the results for each antigen are pooled for measurements on both clones.

**Monoclonal Antibodies and Antibody Fragments:** The cell line HB27, producing a monoclonal anti-H-2L<sup>d</sup>, anti-H-2D<sup>b</sup> antibody, 28-14-8 (16) was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in 600-ml flasks, and the IgG<sub>2</sub> antibody was purified from spent culture medium. Medium, clarified by centrifugation at 300 g for 10 min, was adjusted to pH 8.1 with NaOH and slowly passed over a column of protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C. The column was washed with 5 vol of pH 8.1 0.1 M phosphate buffer and then the bound antibody was eluted with pH 4.5 citrate buffer into tubes containing 1 ml of 1 M Tris buffer, pH 8.5 per 4 ml eluted fraction. Fab fragments were prepared from the purified antibody by brief digestion with papain (4). Both intact Ig and Fab of 28-14-8 were conjugated with rhodamine isothiocyanate (Research Organics, Inc., Cleveland, OH) as previously described (4). Conjugates were tested on EL-4 and on transfected and standard L cells. Both labels were specific. They stained H-2D<sup>b</sup>-positive EL-4 and transfected, but not control L cells and this staining was blocked by excess unlabeled antibody.

**Fluorescence Photobleaching Recovery:** Lateral diffusion of labeled L<sup>d</sup> antigens was measured by fluorescence photobleaching and recovery. In this method a spot, a few micrometers in diameter, is defined by a focused, attenuated, laser beam and the diffusion of labeled molecules into the spot is observed in terms of recovery of fluorescence after bleaching the spot with a brief (milliseconds) intense pulse of laser light. The record of fluorescence recovery yields an estimate of the diffusion coefficient of the labeled molecules and of the fraction of all labeled molecules mobile during the time of observation (review in reference 10). Our instrument, which is computer controlled, has been described previously (4). Unless noted, all measurements were made at 18–20°C.

## RESULTS AND DISCUSSION

Cells were usually labeled with tetramethylrhodamine-conjugated 28-14-8 IgG. In one experiment the diffusion coefficients measured with Fab label did not differ from those measured with IgG. No diffusion of IgG-labeled antigens could be detected if labeled cells were incubated with a

TABLE I  
Lateral Diffusion of Wild-type and Mutant L<sup>d</sup> Antigens in the Plasma Membranes of Transfected L Cells

Gene	Residues in cytoplasmic domain	Diffusion coefficient $\text{cm}^2 \times 10^{-9}$	No. of measurements
27	31	1.2 (0.32)*	43 (3)*
911	7	1.0 (0.21)	117 (9)
C48	4	1.5 (0.45)	28 (3)
907	19	0.8 (0.14)	15 (1)

\* Standard error of the mean.

‡ Number of experiments.

polyclonal goat anti-mouse IgG serum, though the cells still appeared ring stained.

The data for cells examined at 18–20°C are summarized in Table I. No significant differences in diffusion coefficients are seen in these data. The diffusion coefficients for all molecules are close to those measured for endogenous H-2K<sup>b</sup> antigens on L cell clone cl1d,  $1.6 \pm 0.1 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$  (4). Even if the difference between C48 and wild-type L<sup>d</sup> was significant it is no more than 1.5-fold between a molecule lacking 27/31 residues of the cytoplasmic domain and lacking half the cluster of basic residues at the end of the transmembrane domain and a molecule with a complete cytoplasmic domain.

The fraction of all labeled molecules that are mobile in the time of an experiment, R, may be calculated from FPR measurements. R ranged from 15 to 60% in different experiments, with a mean of 31% for labeled 27, BAL911 and BAL907 gene products. Again this is the same as previously found for labeled H-2K<sup>b</sup> antigens of cl1d. An average of only 20% of labeled C48 gene products were mobile, but this low value may be due to the lower level of expression of C48 in transfected cells. Nonspecific cell background intensity is ~700 counts per second (cps) in our machine. Specifically labeled C48 cells had intensities of 800–1,800 cps. Cells with other L<sup>d</sup> transfects labeled with intensities of from 1,500 to 5,000 cps. The lower the ratio between specifically bound label and nonspecific fluorescence, the lower will be the apparent mobile fraction. Hence the true values for mobile fractions may be ~40% for C48 and between 30 and 60% for the other cell clones.

We have recently observed that the mobile fraction of

labeled MHC antigens is greatly reduced in several types of human and mouse cells, including L cells, grown to high density (Wier, M., and M. Edidin, unpublished). BAL911 antigens, bearing only 7/31 residues of the cytoplasmic domain are similarly affected by culture density. An average of 34% of labeled antigens were mobile on 71 individual log phase cells, while an average of 11% were mobile on 43 individual high density cells. Indeed, in two experiments, on 18 cells, no recovery of fluorescence (practically,  $R < 10\%$ ) could be observed, though diffusion was detectable if the measured spot was bleached a second time.

Deletion of up to 27 of 31 residues of the cytoplasmic domain of H-2L<sup>d</sup> antigens does not affect either the lateral diffusion coefficient measured for these antigens or the fraction of all antigens free to diffuse in the plane of the membrane. This result is similar to that obtained for lymphocyte membrane Ig. This molecule has only three cytoplasmic residues, lys-val-lys (21), but diffuses at ca.  $2 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$  (3) and appears to interact with the lymphocyte cytoskeleton (30).

We may consider three plausible mechanisms for regulation of lateral diffusion: (a) The small cytoplasmic tail remaining on the truncated antigens may contain sufficient residues, in particular basic amino acids, interact with the cytoskeleton. (b) The MHC antigens may interact with other membrane integral proteins, which in turn interact with the cytoskeleton. (c) Diffusion may be constrained by the effects of the total, high, concentration of membrane proteins on the viscosity of the lipid bilayer (17, 24). This concentration would not change appreciably in transfected cells. There are precedents for all three possibilities, though the first might be ruled out if a completely truncated L<sup>d</sup> gene could be constructed and expressed in transfected cells. The second possibility is suggested by observations that stearylized dextrans, integrated into the cell membrane bilayer by fatty acid tails, diffuse at  $\sim 3 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$  (31). This 10 to 20 times slower than observed for lipid-soluble dyes and 5–6 times slower than the diffusion of stearyl dextrans in synthetic membranes. The result strongly suggests that stearyl dextran molecules interact with one another and with membrane proteins and that these interactions retard diffusion. The third possibility is raised by recent work on the redistribution of intramembrane particles (IMP) in regenerating axon membranes (24). IMP form gradients in the axon membrane which are best explained by diffusion coefficients for the particles of the order of  $10^{-7} \text{ cm}^2 \text{ s}^{-1}$ . This possibility is also raised by earlier work on the effects of protein concentration on lateral diffusion coefficients in synthetic membranes (2, 17).

Whatever the mechanism, it is clear that the bulk of the C terminal domain of an MHC antigen is not required to restrict that antigen's lateral diffusion in the plane of the membrane.

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